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Isolation of a high molecular weight actin-binding protein from baby hamster kidney (BHK-21) cells

(actin crosslinker/cultured cells/microfilament organization)

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ABSTRACT **A high molecular weight protein (HMWP) with properties similar to those of both actin-binding protein (ABP) and filamin has been isolated from cultured baby hamster kidney (BHK-21) cells. The protein was present in an actomyosin-depleted sucrose extract of the cells and was eluted, upon gel chromatography on Sepharose 4B, near the void volume. The subunit migration on sodium dodecyl sulfate/polyacrylamide gels and the amino acid composition of HMWP were similar to those of ABP and filamin. HMWP bound to and crosslinked F-actin from rabbit muscle, as shown by the formation of a gel that was sedimented with low-speed centrifugation. This interaction was insensitive to temperature and low concentrations of calcium ions, although it may depend on the presence of myosin. Observations of thin sections of the actin–HMWP gel revealed crosslinked complexes of laterally aggregated actin filaments. The axial period of the dense crosslinks was 34 nm. The HMWP may be involved in regulation of microfilament organization.**

Actin from a variety of muscle and nonmuscle cells can interact with myosin to form a contractile complex. This complex is regulated by other proteins, which bind to either actin or myosin. Because the ratios of actin to myosin are an order of magnitude higher in nonmuscle cells than in muscle cells (1, 2), functional interactions of actin with proteins other than those involved in the regulation of actomyosin ATPase have also been proposed. Kane showed that actin in sea urchin egg extracts gelled upon warming to 37°C (3). Two polypeptides, of molecular weight 58,000 and 220,000, appeared to be involved in the process. Recently, it has been shown that the lower molecular weight species crosslinks actin to form needles, and that addition of the high molecular weight protein (HMWP) results in gelation of the needles (4). Other HMWPs that interact with actin to produce gelation have also been described. Actin-binding protein (ABP) from macrophages (5) and leukocytes (6) and filamin from smooth muscle (7–9) have similar, but not identical, physical and biological characteristics, such as size (two identical 250,000-dalton subunits), amino acid composition, and their ability to participate with purified actin in a gelation reaction (5–11). Electrophoretic or immunological analyses have resulted in identification of proteins similar to ABP and filamin in extracts of many other cells (12–17), including cultured mammalian cells (18–20). In addition to the high molecular weight actin crosslinkers, several low molecular weight gelation factors have been isolated from *Acanthamoeba* (21).

As part of our investigation on the role of microfilaments in cell motility, we have prepared native microfilaments from cultured mammalian cells (22). Along with actin and myosin bands, sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels to which isolated microfilaments were applied contained a prominent band that had an approximate subunit molecular weight of 250,000. In order to be certain that these cells contained a protein similar to ABP or filamin, and to tentatively identify the native microfilament component, we have isolated a HMWP from baby hamster kidney (BHK-21) cells. BHK HMWP has chemical properties similar to those of ABP and filamin, and induces the gelation and crosslinking of muscle F-actin.

MATERIALS AND METHODS

Cell Culture. Baby hamster kidney (BHK-21/C13) cells were grown in roller bottles and harvested as described (23).

Isolation of BHK HMWP. Actomyosin was prepared as described by Yerna *et al.* (23). The supernatant remaining after actomyosin was pelleted was centrifuged for 3 hr at 100,000 × *g*. Subsequent purification steps were based on procedures described for isolation of gizzard filamin (7, 8). To the clarified supernatant, ammonium sulfate was added to 35% saturation, at pH 7.5. The precipitate was collected and dialyzed against 0.5 mM EDTA/0.2 mM dithiothreitol/50 mM potassium phosphate, pH 7.5 (4B buffer). The sample was applied to a 2.6 × 95 cm column of Sepharose 4B (Pharmacia) equilibrated in 4B buffer. If cells from more than 30 roller bottles were used, the sample was divided so as not to exceed a 30-bottle equivalent of protein per column run. Fractions were collected at a flow rate of 20 ml/hr. Fractions from the descending portion of the large first peak (which included the void volume) were applied to a $NaDodSO_4$/polyacrylamide slab gel to determine which fractions contained the desired protein. Appropriate fractions were pooled, dialyzed against 0.5 mM EDTA/0.2 mM dithiothreitol/20 mM potassium phosphate, pH 7.5 (DEAE buffer), and applied to a 2.6 × 10 cm column of DEAE-cellulose (for 60 roller bottles) (DE-52, Whatman) that had been equilibrated with DEAE buffer. After washing with 100 ml of DEAE buffer, protein was eluted with 500 ml of a linear gradient consisting of 0–0.4 M NaCl in DEAE buffer, at a flow rate of 40 ml/hr. The first major peak (75–110 mM NaCl) was monitored by $NaDodSO_4$/polyacrylamide slab gel electrophoresis to determine which fractions were enriched for HMWP. These fractions were dialyzed against DEAE buffer and concentrated by application to a 1.5 × 3 cm DE-52 column,

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Abbreviations: ABP, actin-binding protein; $NaDodSO_4$, sodium dodecyl sulfate; HMWP, high molecular weight protein; BHK-21, baby hamster kidney cell line 21/c13; S-1, heavy meromyosin subfragment 1.

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followed by elution with 0.3 M NaCl in DEAE buffer. The 10 ml containing the most concentrated HMWP were applied to a 2.6 × 95 cm Sepharose 4B column and eluted with 4B buffer. Fractions across the single symmetrical peak obtained were once again monitored on a slab gel, and the most highly enriched ones were pooled.

Isolation of Rabbit Skeletal Muscle Proteins. Actin and myosin subfragment 1 (S-1) were prepared as described (24). The actin was free of regulatory proteins and the S-1 contained neither actin nor undigested myosin.

Assay of HMWP Binding to Actin. The proteins were dialyzed against 30 mM KCl/0.5 mM $MgCl_2$/0.2 mM ATP/10 mM potassium phosphate, pH 7.0, for several hours. At the concentrations given in the text, aliquots of the proteins were mixed at 4°C and then incubated for 30 min in a 24°C water bath (or other temperature sequence as described). The tubes were then centrifuged for 10 min at 17,400 × g. Supernatants were removed. Pellets were rinsed in 0.5 ml of dialysis buffer at room temperature and centrifuged again. The pellets were then resuspended in sample buffer for $NaDodSO_4$/polyacrylamide gel analysis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Gels were run in 5-mm diameter glass tubes (25, 26) or 1-mm thick slabs (26) and stained for protein or carbohydrate (25). Densitometer traces were obtained at 550 nm with a Zeiss PM6 spectrophotometer.

Protein Determination. Protein concentrations were estimated by the biuret procedure (27) as described (24).

Amino Acid Analysis. Analysis was performed by using a Durrum D-500 analyzer after 24-hr hydrolysis in 6 M HCl at 110°C under reduced pressure. Values obtained for threonine and serine residues were increased by 5 and 10%, respectively, to correct for hydrolysis.

Microscopy. A small piece of an actin-HMWP gel was flattened between a glass slide and coverslip and was observed under a Zeiss Photomicroscope III equipped with polarized light optics and a λ/20 compensator.

For electron microscopy, the sample was fixed with 1% glutaraldehyde and 0.2% tannic acid (28) in phosphate-buffered saline (29) at 24°C for 40 min. It was then rinsed for 15 min with several changes of phosphate-buffered saline and for 1 min at 4°C with 50 mM KCl/5 mM $MgCl_2$/50 mM sodium phosphate, pH 6.0. The sample was postfixed with 2 mM OsO_4 in the pH 6.0 buffer at 4°C for 10 min (30), rinsed, and left in distilled H_2O for 1 hr at 24°C. Dehydration, embedding, sectioning, and staining were as described (24). Sections were photographed on a Philips 201C microscope operated at 60 kV. Measurements were made from negatives and calibrated with a grating replica.

RESULTS

Actomyosin-depleted sucrose extracts of BHK-21 cells contained a protein whose molecular weight was appropriate for a high molecular weight actin-binding protein (Fig. 1). This fraction was therefore used as a starting material. The ammonium sulfate fractionation and chromatographic procedures described resulted in a fraction, 80–90% of which migrated on $NaDodSO_4$/polyacrylamide gels with an approximate molecular weight of 250,000 (Fig. 1). A band with the same relative mobility was present on a gel containing whole BHK-21 cell homogenate, in an amount similar to that of myosin heavy chain (not shown). The recovery from 60 roller bottles (30 ml of packed cells) was 2 to 4 mg of HMWP.

BHK HMWP was soluble at high (0.6 M KCl) and low (30 mM KCl/10 mM phosphate) ionic strength at neutral pH. It contained no detectable carbohydrate, as determined by the absence of periodic acid/Schiff-staining material on $NaDodSO_4$/polyacrylamide gels loaded with 60 μg of HMWP. Under these conditions, 50 μg of BHK-21 cell homogenate contained several periodic acid/Schiff-positive bands (results not shown). The amino acid composition of BHK HMWP was similar to that of ABP and filamin (Table 1).

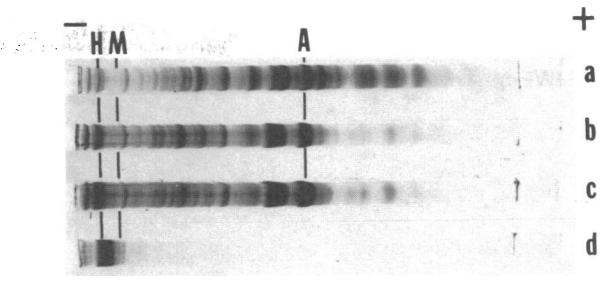

FIG. 1. $NaDodSO_4$/polyacrylamide gel (25) analysis of samples during purification of the HMWP. Lane a, supernatant after precipitation of actomyosin; lane b, sample applied to first Sepharose 4B column; lane c, sample applied to first DEAE-cellulose column; lane d, purified HMWP. Migration positions of HMWP (H), myosin heavy chain (M), and actin (A) are shown. Each gel contained 25 μg of protein.

When isolated BHK HMWP was mixed with purified rabbit skeletal muscle F-actin, a gel formed within 60 sec. The gel did not occupy the entire solution volume, but appeared as a light-scattering bubble-trapping mass suspended in the clear solution. This mass decreased in size during a 1-hr incubation in a fashion similar to the reported coagulation of filamin–actin complexes (11).

The interaction of BHK HMWP with actin was studied by mixing the proteins under various conditions, collecting the resulting gel, and analyzing the pellet by electrophoresis on $NaDodSO_4$/polyacrylamide. An example of such an experiment is shown in Fig. 2. Actin and HMWP were combined at 4°C in the ratios shown, incubated at 24°C for 30 min, and centrifuged at 24°C. The gelled pellets contained both proteins (Fig. 2, lanes G–K). Very little actin (Fig. 2, lane F) and no HMWP (Fig. 2, lane E) was pelleted in control tubes. Incubation and centrifugation at 4°C (Fig. 2, lane D) or incubation at

Table 1. Amino acid composition of BHK HMWP: Comparison with filamin and ABP

Residue	Amino acid composition, mol %			
	BHK HMWP	Mammalian filamin*	Avian filamin†	Macrophage ABP‡
Asp	8.9	9.2	7.6	8.7
Thr	6.6	6.4	5.3	6.2
Ser	7.5	6.8	6.8	6.8
Glu	11.0	10.8	9.5	11.4
Pro	8.0	8.7	8.0	7.1
Gly	10.7	12.0	12.5	11.8
Ala	6.9	7.7	9.3	7.4
Val	9.6	9.8	10.0	8.5
Met	1.3	1.1	0.8	1.3
Ile	4.4	4.1	3.3	4.4
Leu	5.8	6.0	6.0	6.2
Tyr	2.8	2.7	2.6	3.1
Phe	2.8	2.2	2.9	3.2
His	2.1	2.1	2.3	2.2
Lys	6.0	6.4	4.8	6.0
Arg	3.3	3.5	5.5	4.1

* Ref. 9.
† Ref. 7.
‡ Ref. 10.

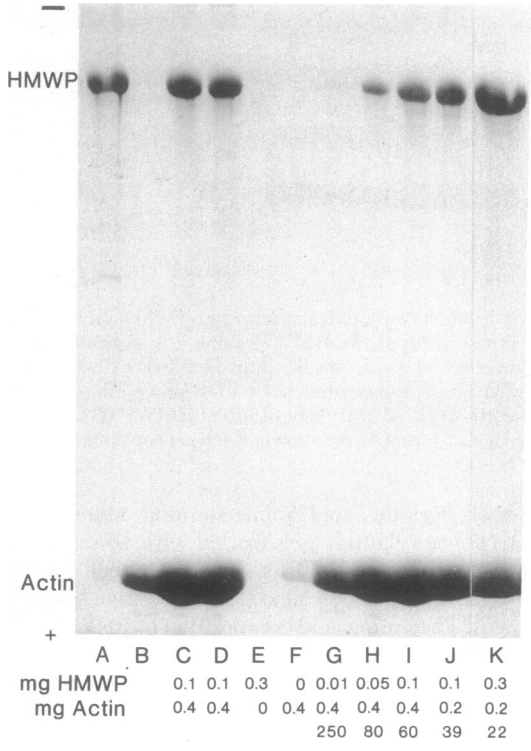

FIG. 2. The gelation process was assayed by cosedimentation followed by analysis on $NaDodSO_4$/7.5% polyacrylamide (26). Proteins were mixed at 4°C in the amounts shown below each well (C–K), in 30 mM KCl/0.5 mM $MgCl_2$/0.2 mM ATP/10 mM potassium phosphate, pH 7.0. Total volume in each tube was 0.5 ml. Samples E–K were incubated for 30 min at 24°C and then centrifuged for 10 min at 17,400 × *g*. Sample D was incubated for 30 min at 4°C and centrifuged at 4°C. Sample C was incubated for 30 min at 24°C and 30 additional min at 4°C and then centrifuged at 4°C as above. The supernatants were aspirated, pellets were washed, and 7% of each sample was applied to the slab gel. Throughout the concentration range tested, actin and HMWP cosedimented (G–K) under conditions in which, separately, neither protein alone was pelleted (E and F). The gelation process was temperature insensitive (C and D). The migration positions of HMWP and actin are shown. Samples G–K were run on 5-mm diameter tube gels (26) at a protein load equivalent to 1/8th of that shown above. By densitometry, rough estimates of the actin/HMWP molar ratios were obtained, and the values are shown below the appropriate wells.

24°C followed by incubation and centrifugation at 4°C (Fig. 2, lane C) resulted in pellets identical to the one obtained for 24°C incubation and centrifugation (Fig. 2, lane I). Thus, under the conditions of the assay, gelation was temperature independent.

The complex sedimenting with low speed centrifugation was formed over a wide range of actin/HMWP ratios. For example, a complex was formed that contained a barely detectable amount of HMWP, on a gel that was overloaded for actin (Fig. 2, lane G). On the other hand, actin was capable of binding a large amount of HMWP (Fig. 2, lane K).

Preliminary experiments in which rabbit muscle S-1 was added to the actin before mixing with HMWP have indicated that the actin–HMWP interaction is partially inhibited by S-1. Although gelation occurred in these samples, less HMWP was found in the pellets in the presence than in the absence of S-1. The same series of preliminary experiments suggested that cosedimentation of actin with purified HMWP occurs regardless of the presence or absence of 1 mM ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) in the assay solution (muscle and nonmuscle actomyosin are regulated in this range of free calcium concentration, ref. 23).

When observed by polarized light microscopy, the actin–HMWP gel was anistropic (Fig. 3 *a* and *b*). This demonstration of molecular order prompted us to examine the ultrastructure of the gel. Thin sections of the gel revealed crosslinked arrays of actin (Fig. 3 *d*, *e*, and *f*). However, only a small proportion of the actin in the gel (10% was a very rough estimate from observations of thin sections) was involved in these arrays. Within the arrays, laterally aggregated actin filaments were joined by electron-dense material. The axial spacing of the crosslinks was measured as 33.9 ± 0.9 nm (SD, $n = 147$). Careful examination of thin sections of a pellet of the actin used for these experiments revealed uniform actin structure without any evidence for crosslinked arrays (Fig. 3*c*). Thus the crosslinking structures probably contained HMWP.

DISCUSSION

HMWPs that interact with actin have been implicated in cytoplasmic consistency changes (3, 10, 11, 13–15, 19). Macrophage ABP and smooth muscle filamin have been isolated and appear to be similar (7–10). The present investigation has made possible the comparison of HMWP isolated from cultured fibroblasts with ABP and filamin. We have adopted the general term "HMWP" (19) to avoid additional terminology; precise comparison of the three proteins awaits amino acid sequence determination to unequivocally demonstrate their proposed identity (9, 31).

BHK HMWP was chemically similar to ABP and filamin on the basis of (*i*) its subunit and native molecular weight as determined by $NaDodSO_4$ electrophoresis and gel permeation chromatography, (*ii*) its solubility, (*iii*) the absence of carbohydrate, and (*iv*) its amino acid composition. It bound to actin, resulting in gel formation. Additional similarity to purified filamin was shown by the insensitivity of gelation to temperature and calcium concentration (cf. ref. 11). On the other hand, gelation of purified ABP and actin, or of extracts from several cell types, is temperature and calcium sensitive (10, 13–15). The different observations may be due to changes in the HMWP during isolation, such as partial denaturation or proteolysis, or to loss of required regulatory factors. Attempts to obtain initial enrichment of BHK HMWP by precipitation in a complex with actin (as in ref. 5) were unsuccessful. Thus we used the somewhat lengthier procedures described for filamin isolation (7, 8).

The role of actin-gelling proteins in cell movement is not yet completely understood. It would be useful to know the ultrastructural arrangement of actin filaments that are in the crosslinked state. Our micrographs have demonstrated that HMWP can form regular arrays along laterally aggregated F-actin. The axial period of the crosslinks is close to the half pitch of the actin helix, suggesting that HMWP recognizes a specific aspect of the F-actin structure. The ability of HMWP to occupy closely spaced sites on F-actin is consistent with a possible competitive interaction between HMWP and myosin, with actin. This competition has been discussed by others (12, 14, 31–34), appeared in our preliminary results with S-1, and was inherent in the preparative scheme. Perhaps actin–HMWP complexes represent the cyto*skeletal* form of actin, in contrast to the *contractile* actin that complexes with myosin.

On the structural level, these differences may be reflected in the two interconvertible states of microfilament organization that have been described, namely bundles and meshworks (see ref. 35). It is of interest to determine the possible role of the HMWP in microfilament bundle and meshwork formation. Experiments that combine examination of the ultrastructure of

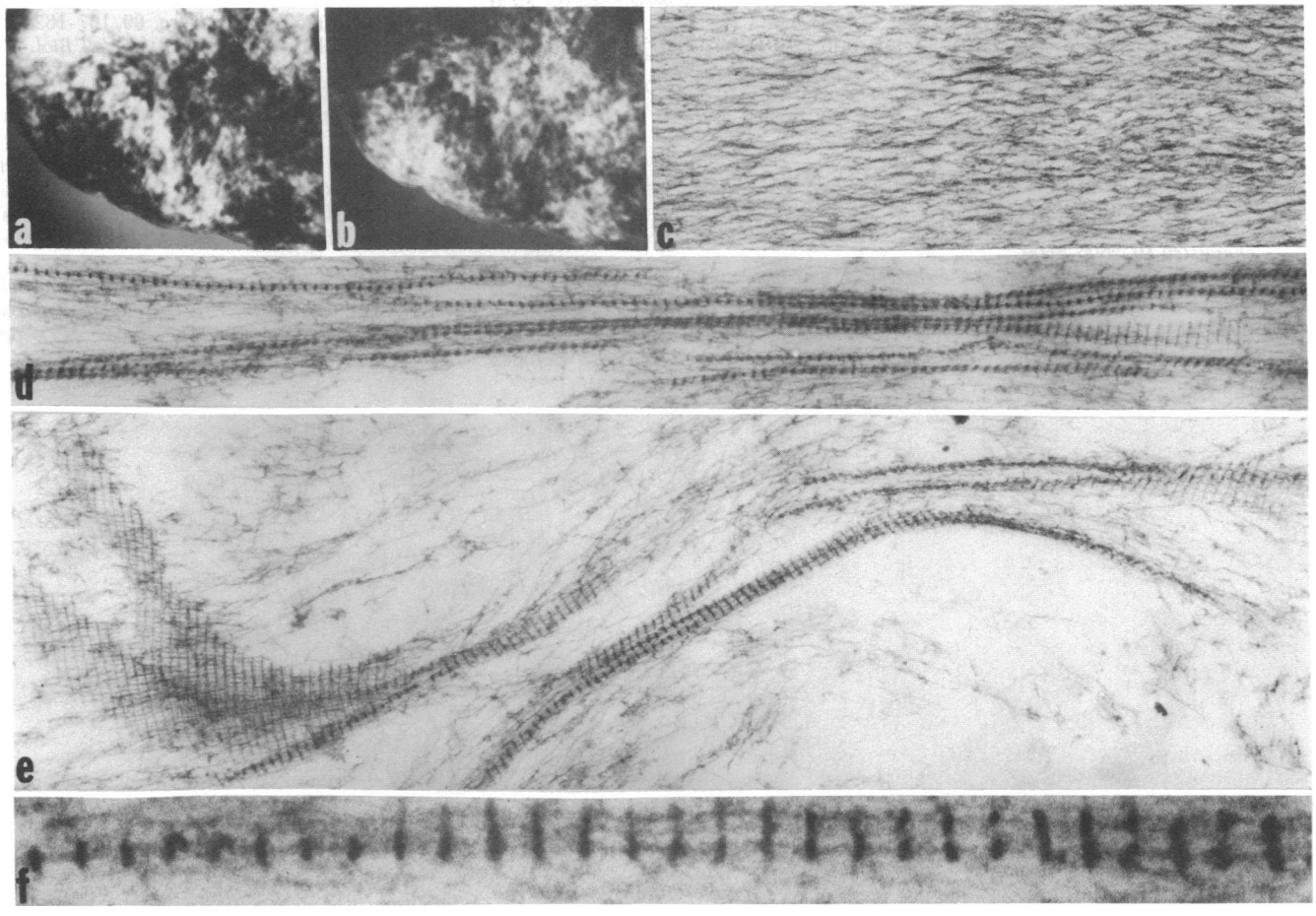

FIG. 3. Microscopic analysis of actin–HMWP gel. A small piece of a gel (formed as sample I in Fig. 2) was flattened under a coverslip and observed in polarized light (*a*, *b*) at opposite compensator settings. (×220.) (*d*, *e*, and *f*) Electron micrographs of thin sections of an actin–HMWP gel (prepared as sample I in Fig. 2). Superimposed over a background of normal-appearing actin were complexes in which regularly spaced crosslinks were arrayed perpendicular to the long axes of F actin. These complexes usually appeared as long narrow strands (*d*), and sometimes, perhaps due to the plane of section, they appeared as nets (*e*). A ladder image was seen at higher magnifications. (*f*) The actin from which the gel was made appeared homogeneous, and displayed no crosslinked complexes (*c*). (*c–e*, ×38,500; *f*, ×190,000.)

actin-HMWP gels formed *in vitro* with elucidation of mechanisms for regulating crosslinking should contribute to our understanding of *in vivo* changes in microfilament organization. These studies can be aided by the ability to work with purified proteins. This report has shown that a gel-inducing protein from cultured fibroblasts is similar to such proteins isolated from other cells. It remains to be determined which factors control the cytoskeletal organizational changes in which this protein may function.

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